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**Figure 1**

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## RESHAPING MONOCLONAL ANTIBODIES AGAINST JUNIN AND VACCINIA VIRUSES

By William J Harris, Anita A Hamilton, Philip R Tempest, Marie L Fernie,  
Susan I King, Patricia Bremner and Frank J Carr

### 1. INTRODUCTION

This is the final report of the project entitled "The Provision of Human Monoclonal Antibodies for Therapy and Passive Immunisation by Reshaping Rodent Monoclonal Antibodies". Two mouse monoclonal antibody producing cell lines were supplied by USAMRIID, together with materials for assaying the antibodies produced. The first was against junin, an arenavirus, the second against vaccinia, an orthopoxvirus. These mouse antibodies have been converted to human antibodies by the application of "antibody reshaping" technology pioneered by Dr G Winter (1) to which Scotgen holds a non-exclusive licence.

The advent of hybridoma technology in 1975 (2) held great promise for the use of monoclonal antibodies in the diagnosis and treatment of human disease, for instance as "magic bullets". However, there are problems associated with administering mouse antibodies to human subjects. A human anti-mouse antibody response results in unacceptable anaphylactic reactions which prevent repeated administration, or in very rapid clearance of the antibody from the body. Initially it was confidently expected that the technology could be simply extended to make human monoclonals, but this has proved not to be the case. There are difficulties in finding appropriately immunised human donors and suitable fusion partners for the antibody producing cells. *In vitro* immunisation techniques have been tried, but only low affinity IgM antibodies are generally produced.

The first advance in the conversion of mouse antibodies to human was the development of "chimeric" antibodies - hybrids with murine variable regions linked to human constant regions (3). This is further refined in the Winter technology where only the specific sequences which determine antigen binding, the "complementarity determining regions" or CDRs, are taken from the mouse and grafted onto human variable region frameworks.

### 2. MATERIALS AND METHODS

#### 2.1 Cell Lines

The mouse hybridoma cell lines provided by USAMRIID were Y-GD01-AG02-1 (YGD) and VVI-10F5-4-1 (VVI) which secrete antibodies against junin and vaccinia viruses respectively. Growth of cells, antibody isotyping and RNA isolation were described fully in the mid-term report.

#### 2.2 Enzymes

All restriction and modification enzymes were obtained from Gibco BRL unless otherwise stated and were used with appropriate buffers according to the manufacturer's instructions.

### 2.3 Oligonucleotides

Oligonucleotides were made on an Applied Biosystems 381 DNA synthesiser and were generally used without further purification. The primers for cDNA synthesis were as described by Orlandi *et al* (4).

VH1FOR 5'TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG3',

VH1BACK 5'AGGTSMARCTGCAGSAGTCWGG3',

VK1FOR 5'GTT AGATCTCCAGCTTGGTCCC3',

VK1BACK 5'GACATTCAGCTGACCCAGTCTCCA3',

VK2BACK 5'GACRTTCAGCTGACCCAGGMTGMA3',

VK3BACK 5'GACATTCAGCTGACCCA3'

where M = C or A, S = C or G, and W = A or T

### 2.4 cDNA synthesis

Reactions consisted of 10 ug RNA, 0.4 uM VH1FOR or VK1FOR primers, 250 uM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH7.5, 75 mM KCl, 3.0 mM MgCl<sub>2</sub> and 27 units of RNase inhibitor (Pharmacia) in a total volume of 50 uL. The samples were heated to 70°C for 10 minutes and cooled slowly to 42°C over 30 minutes. 100 units of Moloney murine leukaemia virus reverse transcriptase were added and the incubation at 42°C continued for 1 hour.

### 2.5 Amplification of VH and VK DNA by PCR

For amplification of VH DNA the reactions consisted of 10 uL of VH cDNA/RNA hybrid, 0.5 uM VH1FOR and VH1BACK primers, 200 uM of each of dATP, dCTP, dTTP and dGTP, 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) NP40 and 1 unit of Taq DNA polymerase (Cetus). For amplification of VK DNA the VK cDNA/RNA hybrid and primers VK1FOR and either VK1BACK or VK2BACK or VK3BACK are substituted. The samples were subjected to 25 thermal cycles of 94°C, 1 minute; 50°C, 1 minute; 72°C, 2 minutes; finishing with 5 minutes at 72°C.

### 2.6 Cloning and sequencing VH and VK DNA

Amplified VH DNA was purified from 1.5% low melting point agarose gels by Elutip-d column chromatography (Schleicher and Schuell) and digested with *Pst*I and *Bst*EII. The *Bst*EII site was made blunt-ended by filling in with Klenow fragment of DNA polymerase I and the DNA cloned into M13mp18 and M13mp19 cut with *Pst*I and *Sma*I. The amplified VK DNA was purified in the same way, cut with *Pvu*II and *Bgl*II and cloned into M13mp18 and M13mp19. DNA from the clones was sequenced by the dideoxy method (5) using Sequenase (United States Biochemicals).

### 2.7 Mutagenesis of human VH and VK genes

Oligonucleotide site-directed mutagenesis of the human VH and VK genes was based on the method of Eckstein (6). To 5 ug of VH or VK single-stranded DNA in

M13 (M13VHPCR1 or M13VKPCR2), a two-fold molar excess of each of the three VH or VK phosphorylated oligonucleotides encoding the 3 mouse CDR (complementarity determining region) sequences was added. The primers were annealed to the template by heating to 70°C for a few minutes and slowly cooling to 37°C. The annealed DNA was extended with Klenow fragment of DNA polymerase I in a reaction mixture containing T4 DNA ligase and 0.5 mM of each of dATP, dGTP, dTTP and 2'-deoxycytidine 5'-O-(1-thiotriphosphate) (thiodCTP, Pharmacia) in appropriate buffer. The mixture was incubated at 16°C for 16 hours then the DNA was ethanol precipitated and digested with *Nci*I for 90 minutes. This enzyme nicks the parental strand, but leaves the newly-synthesised strand containing the thiodCTP intact. The parental strand was removed by digestion with exonuclease III then the DNA was repaired with DNA polymerase I and T4 DNA ligase. The DNA was transformed into *E. coli* TG1 made competent by the method of Simanis (7). Single stranded DNA was prepared from individual clones and sequenced. If only single or double mutants were found, these were subjected to further rounds of mutagenesis with the appropriate oligonucleotides until the triple CDR mutants were obtained.

## 2.8 Mutagenesis by PCR

For improved efficiency of mutagenesis a modification involving PCR was introduced. The initial synthesis of the mutant strand was with Klenow fragment of DNA polymerase I and DNA ligase as described above or with T7 DNA polymerase (Pharmacia) and DNA ligase for 1 hour at 37°C. An additional primer at the 3' end of the gene was included and dCTP was substituted for thiodCTP. An asymmetric PCR reaction was performed with a single primer at the 5' end to selectively copy the mutant strand, then the amplification continued with primers at both ends of the gene to produce a double stranded DNA. This was cloned into M13 as a *Hind*III to *Bam*HI fragment for sequencing as before.

To obtain mutations at single sites, for instance for the production of "2nd generation" reshaped constructs, the technique of mutagenesis by overlapping PCR reactions was employed (8).

## 2.9 "Sticky feet"-directed mutagenesis

"Sticky feet"-directed mutagenesis (9) is a technique for precisely cutting and pasting two DNA sequences without using restriction enzymes. The DNA fragment to be transferred is amplified by PCR using tagged (sticky-foot) primers, the 5' tags being complementary to the desired points of insertion in the vector and the "forward" primer being phosphorylated. The PCR product was purified from a low melting point gel as before. Single-stranded template DNA (in M13) and the primer were annealed in a mixture containing PCR buffer, 0.5 mM dNTPs and Taq polymerase. T4 DNA ligase and T7 DNA polymerase were added in appropriate buffer and the reaction incubated at 20°C for 30 minutes. Aliquots were used to transform *E. coli* TG1 as previously described and individual clones isolated and sequenced.

## 2.10 Expression vectors

The reshaped VH and VK genes were expressed in the vectors pSVgpt and pSVhyg shown in figures 1 and 2, which were obtained from Dr G Winter, MRC Laboratory of Molecular Biology, Cambridge. The reshaped VH gene, together with the immunoglobulin heavy chain promoter, appropriate splice sites and signal peptide



sequences was excised from M13 by digestion with HindIII and BamHI and cloned into the heavy chain expression vector containing the murine heavy chain immunoglobulin enhancer, the gpt gene under control of the SV40 promoter/enhancer for selection in mammalian cells and genes for replication and selection in E. coli. A human IgG1 constant region was added as a BamHI fragment and the correct orientation selected. The construction of the light chain vector was the same except that the gpt gene is replaced by the gene for hygromycin resistance (hyg) and a human kappa constant region is included in the plasmid.

### **2.11 Transfection into myeloma cells**

10 ug of the heavy chain expression vector DNA and 20 ug of the light chain expression vector DNA was cut with PvuI to linearise then precipitated with ethanol and redissolved in 25 uL of water. The recipient cell line used was YB2/0, a non-immunoglobulin producing rat myeloma, ATCC No CRL 1662, and is grown in Dulbecco's Modified Eagle's Medium (Gibco BRL or Sigma) supplemented with 10% fetal calf serum and antibiotics (DMEM). Approximately 10<sup>6</sup> YB2/0 cells (one 75 cm<sup>2</sup> flask grown not quite to saturation) were removed from the substrate by mechanical agitation, harvested by centrifugation and resuspended in 0.5 ml of DMEM. The digested DNA was added and the cells transferred to a cuvette and placed on ice for 5 minutes. A single pulse of 170 volts, 960 ufarads was administered (Genepulser, BioRad). After a further 20 minutes on ice the cells were replaced in a flask with 20 ml of DMEM and allowed to recover for 48 hours. At this time the cells were distributed into a 24-well plate in selective medium (DMEM with 0.8 ug/mL mycophenolic acid and 250 ug/mL xanthine). After 3 to 4 days the medium was changed for fresh selective medium so that dead cells were removed. Colonies of transfected cells were visible with the naked eye 10 to 14 days later.

### **2.12 Assay for human antibody**

Capture antibody, goat anti-human IgG, gamma chain specific (Sera-Lab) was diluted to 5 ug/mL in 50 mM carbonate buffer pH9.6 and used to coat polystyrene ELISA plates (Dynatech Immulon 1), 100-200 uL per well, overnight at 4°C or for a few hours at 37°C. The wells were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST), the last wash being left in the wells for 20 minutes. The wells were filled with 50-200 uL of the culture medium to be screened and incubated for 1 hour at 37°C. After washing 3 times with PBST, the reporter antibody, peroxidase-conjugated goat anti-human IgG, gamma chain specific (Sera-Lab) or peroxidase-conjugated goat anti-human kappa chain (Sera-Lab) was added and the plate incubated for 1 hour at room temperature. The plate was washed 3 times with PBST as before then the colour was developed. Substrate buffer was prepared by mixing 100 mM citric acid and 100 mM disodium hydrogen phosphate to pH5.0. 34mg of o-phenylenediamine was dissolved in 100 mL of this and 10 uL of 30% hydrogen peroxide added just before use. 200 uL was dispensed per well and incubated at room temperature in the dark. The reaction was stopped by addition of 50 uL per well of 12.5% (v/v) sulphuric acid and the absorbances were read at 492 nm.

### **2.13 Anti-junin ELISA**

An inactivated junin virus preparation was provided by USAMRIID. This was diluted 1:100 in 50 mM carbonate buffer pH9.6 and used to coat polystyrene ELISA plates (Dynatech Immulon 1), 200 uL per well, at 4°C overnight or at 37°C for 1

to 2 hours. After washing 3 times with PBST, the primary (test) antibodies were applied using PBST as diluent and incubated at 37°C for 1 hour. The wells were again washed with PBST then the secondary antibody, diluted 1:1000 in PBST, added and the incubation continued for 1 hour. This was peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-human IgG or biotinylated goat anti-mouse IgG or biotinylated goat anti-human IgG (Sera-Lab). When biotinylated secondary antibody was used the final detection reagent was streptavidin conjugated to peroxidase (Sera-Lab). The colour was developed with o-phenylenediamine substrate as for the ELISA for human antibody (see 2.12).

#### **2.14 Anti-vaccinia ELISA**

A sucrose gradient-purified preparation of vaccinia virus, inactivated by gamma irradiation, was provided by USAMRIID. This was diluted 1:100 or 1:50 in PBS and used to coat PVC ELISA plates (Dynatech Microelisa), at 50 µL per well, for 1 to 2 hours at 37°C. The wells were blocked for 1 hour at 37°C with 3% bovine serum albumin (BSA) in PBS. After washing twice with PBS containing 0.02% Tween-20 (PBST2) the primary (test) antibody preparations were applied, diluted in 2% BSA in PBS. Incubation was for 1 to 2 hours at 37°C then the wells were washed 4 or 5 times with PBST2 and the secondary antibody added. This was either peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-human IgG (Sera-Lab). Colour was developed with o-phenylene diamine substrate as for the ELISA for human antibody (see 2.12).

#### **2.15 Immunoblotting**

10 µL samples of vaccinia antigen preparation were mixed with an equal volume of Laemmli sample buffer (10) and 0.1 volumes of 1.0 M iodoacetamide and loaded on a 10% SDS-PAGE gel. Rainbow markers (Amersham International) and samples of the antibody preparations were made up similarly and included on the gel as controls. The gel was transferred to nitrocellulose at 145 mA constant current for 1 hour using a Sartoblot II apparatus (Sartorius). The efficiency of transfer was monitored by observation of the Rainbow markers. The filters were blocked overnight at 4°C with 10 mM Tris-HCl, 0.9% NaCl (w/v) pH7.4 (Tris/saline) containing 3% powdered milk (w/v). The primary (test) antibody was added to 1 µg/mL and incubated for 1 hour at room temperature. After several washes with Tris/saline the secondary antibody, biotin-conjugated goat anti-human IgG or anti-mouse IgG (Sera-Lab), diluted 1:1000 in Tris/saline, was added and the incubation continued for 1 hour. The filters were again washed thoroughly with Tris/saline then avidin-alkaline phosphatase (Sigma), diluted 1:1000 in Tris/saline, was added and incubated for 30 minutes. After again washing with Tris/saline the filters were given a final wash with alkaline phosphatase substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 5.0 mM MgCl<sub>2</sub>, pH9.5) then the substrate applied. 40 µL of 75 mg/mL Nitro blue tetrazolium (NBT) in 70% N,N'-dimethylformamide followed by 40 µL of 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in N,N'-dimethylformamide was added to 10 mL of substrate buffer with mixing in between. The colour development was stopped by washing several times with water.

#### **2.16 Purification of antibody**

For small scale purification, cells were grown in 150 cm<sup>2</sup> flasks in selective medium. Conditioned medium was harvested by centrifugation. 0.1 volumes of 1.0 M Tris-HCl, pH8.0 and 0.5 mL Protein A-agarose (Boehringer Mannheim) were added. This was stirred overnight at room temperature then passed through a

small disposable column. This was washed with 10 column volumes of 100 mM Tris-HCl, pH8.0 and 10 column volumes of 10 mM Tris-HCl, pH8.0 and eluted with 100 mM glycine buffer, pH3.0. 500 uL fractions were collected into microfuge tubes containing 50 uL 1.0 M Tris-HCl, pH8.0.

For larger scale preparations, cells were grown in either roller bottles or a small-scale fermenter, generally in non-selective medium. The cells were removed either by centrifugation or filtration and the conditioned medium concentrated by cross-flow filtration through a 100 kD cut-off filter (Sartorius) or 30 kD cut-off filter (Flowgen). The concentrate was passed through a Pro-Sep A column (Bioprocessing Ltd) and eluted as before. Antibody-containing fractions were pooled and dialysed against PBS.

The concentration of the antibody preparations was determined by measuring the absorbance at 280 nm, using the formula, 1 OD unit = 0.8 mg/mL (11). Samples were checked by running on 10% SDS-polyacrylamide gels.

### **3. RESULTS**

#### **3.1 ANTI-JUNIN ANTIBODY**

##### **3.1.1 Status at contract mid-term**

By the mid-term point the junin project had progressed to the stage of transfecting the first reshaped junin antibody construct into myeloma cells. After confirming that they were secreting an antibody of the correct isotype, IgG2a, kappa, RNA was extracted from the mouse Y-GD01 hybridoma cells. cDNA was synthesised and the Y-GD01 VH and VK genes amplified by PCR and cloned into M13 for DNA sequencing. The VH and VK sequences fell into Kabat subgroups IIB and V respectively (figure 3) (12). The Y-GD01 CDR sequences were transplanted into human frameworks, NEWM for the heavy chain and REI for the light chain, and the reshaped VH and VK genes cloned into expression plasmids. These were cotransfected into YB2/0 cells and cell clones resistant to mycophenolic acid were obtained. However, none of these were positive for production of human antibody. The expression plasmids were therefore checked by restriction enzyme digestion and sequencing of the variable regions, revealing that a GC base pair in CDR1 of VK had been deleted during the original site-directed mutagenesis. The resultant reading frame shift would give a non-functional kappa chain and, as heavy chains alone are not secreted, prevent antibody production. An alternative reshaped M13 VK clone was available and this was comprehensively sequenced to ensure the absence of undesirable mutations in the variable region.

##### **3.1.2 Transfection into myeloma cells**

The construction of the kappa chain expression plasmid was repeated using the alternative reshaped M13 clone described above and the sequence was checked again in the final vector. Approximately  $10^7$  YB2/0 rat myeloma cells were co-transfected by electroporation with 10 ug linearised pSV<sub>gpt</sub>HuVHHuLgI (reshaped VH) and 20 ug linearised pSV<sub>hyg</sub>HuVHHuCK (reshaped VK). After 2 days, the cells were distributed into 24 well plates in medium containing mycophenolic acid and xanthine to select for the *gpt* gene carried on the heavy chain plasmid. It is not necessary to perform a double selection as it has been found that all mycophenolic acid-resistant clones have also taken up the light chain plasmid. After a further 10 days mycophenolic acid resistant colonies were present in all wells.

### 3.1.3 ELISA screening for reshaped antibody production

Microtitre plates coated with goat anti-human IgG were used to immobilise any human antibody in the medium from the wells. This was then detected using peroxidase-conjugated goat anti-human IgG or peroxidase-conjugated goat anti-human kappa. All 24 culture supernatants were positive for both human IgG and human kappa.

### 3.1.4 Purification of reshaped antibody

Transfected clones giving the highest ELISA readings were expanded and the culture medium pooled. Antibody was purified by protein A agarose affinity chromatography. 375 ug of reshaped antibody was isolated from 250 ml of medium. This represents a yield of  $1.5\text{ug}/10^6$  cells/ml.

### 3.1.5 ELISA of binding to junin virus

The reshaped anti-junin antibody was tested for binding to inactivated junin virus. A typical ELISA comparing antigen binding of the murine antibody and the reshaped antibody (HuYGD) is shown in figure 4. The binding appears more or less equivalent, but an exact comparison is not possible because different reporter antibodies (anti-mouse IgG or anti-human IgG) are used.

### 3.1.6 Virus neutralisation by reshaped antibody

150 ug of the reshaped antibody preparation was sent to Dr Alan Schmaljohn at USAMRIID. Experiments at USAMRIID showed that the reshaped YGD antibody bound to junin virus-infected cells as measured by indirect immunofluorescence. The reshaped antibody also neutralised virus infectivity *in vitro*, but was approximately 10-fold less effective in this assay than the original murine monoclonal antibody, presumably reflecting a decrease in binding efficiency (results not included in this report).

### 3.1.7 Construction of chimeric antibody plasmids

The appropriate control antibody for these ELISAs is the hybrid chimeric antibody with murine variable regions linked to human constant regions. The chimeric VH gene was constructed by first removing the internal PstI site from YGD MuVH (obtained from PCR amplification of cDNA) by *in situ* mutagenesis, then cloning YGD MuVH into M13VHPCR1 as a PstI to BstEII fragment.

The chimeric VK gene was constructed by cloning YGD MuVK cut with PvuII and BglII into M13VKPCR1 cut with PvuII and BclI. The latter restriction enzyme site has been removed from the reshaped constructs as it causes the inclusion of an abnormal amino acid in the antibody chain.

The HindIII to BamHI fragments containing the VH and VK variable regions were inserted into the expression vectors pSV<sub>got</sub> and pSV<sub>hyg</sub> with appropriate constant regions as for the reshaped antibody genes.

### 3.1.8 Production of chimeric and "mix and match" antibodies

To investigate whether the reduced affinity (shown by reduced virus neutralisation) of the reshaped YGD (HuYGD) is due to the reshaped heavy or reshaped light

chain, both chimeric and hybrid chimeric/reshaped antibodies were produced. The chimeric and reshaped expression vectors were cotransfected into YB2/0 cells by electroporation in the following combinations:- MuVH/MuVK, MuVH/HuVK and HuVH/MuVK. Cell clones resistant to mycophenolic acid were assayed for production of antibodies containing human constant regions. Chimeric and hybrid chimeric/reshaped antibodies were purified from pooled clones as before.

### **3.1.9 Construction of "2nd generation" reshaped VH**

From previous experience in the laboratory, it was anticipated that the heavy chain variable region was most likely to be important in determining affinity. As data from USAMRIID on *in vitro* virus neutralisation indicated that the affinity of the reshaped antibody was reduced, the construction of a "2nd generation" reshaped VH was initiated.

A comparison of the murine and reshaped VH and VK genes is shown in figure 5. Only residues close to the CDRs are likely to be important, except for certain residues in VH framework 3. The residue at VH position 72 (Kabat residue 71) has been implicated in packing against CDR 2 (13) and other framework 3 residues may form a 4th CDR type loop. As most of the residues close to CDRs are similar if not identical in both murine and reshaped VHs, attention was concentrated on framework 3.

Leu 71 Val 72 were changed to Thr71 Ala 72 by *in situ* mutagenesis of the reshaped VH gene and this "TA" VH cloned into the expression vector. This HuVHTA and the reshaped VK were cotransfected into YB2/0 cells and transfectants secreting human antibody selected as before. 2nd generation HuVHTA-YGD antibody was purified from pooled transfected clones as before. A hybrid antibody was also produced by cotransfecting the HuVHTA plasmid and the MuVK (chimeric) plasmid.

### **3.1.10 Analysis of binding of chimeric, mix and match, and 2nd generation reshaped antibodies by ELISA**

The murine, chimeric, hybrid chimeric/reshaped and the "2nd generation" reshaped antibodies were tested for binding to inactivated junin virus. Figure 6 shows an ELISA comparing binding by the chimeric and the first reshaped antibody. In this assay bound antibody was detected with biotinylated goat anti-human IgG, followed by peroxidase-conjugated streptavidin. There was little, if any, difference in antigen binding by the two antibodies.

In a similar ELISA (figure 7) antigen binding by the 2nd generation reshaped (HuVHTA-YGD), the 1st generation reshaped and the murine antibodies was compared. The HuVHTA-YGD antibody showed a clear increase in affinity.

This finding is confirmed by the results shown in figure 8, an ELISA comparing binding by two hybrid antibodies, HuVH/MuVK and HuVHTA/MuVK, with the 1st and 2nd generation reshaped, HuVH/HuVK and HuVHTA/HuVK, and the original murine antibody. The 2nd generation reshaped VH, combined with either the reshaped VK or murine (chimeric) VK shows increased binding over the 1st generation reshaped or original murine antibodies, which are equivalent in this assay. A small quantity of the HuVHTA-YGD antibody was sent to USAMRIID for testing in virus neutralisation experiments.

### **3.1.11 Large scale preparation of reshaped and chimeric antibodies**

Quantities of reshaped HuVHTA-YGD and chimeric anti-junin antibodies were prepared for shipment to USAMRIID. After removal of cells, 7 litres of conditioned medium was concentrated by cross-flow filtration through a 30 kD cut-off filter. The antibody was purified by protein A affinity chromatography and antibody fractions pooled and dialysed against phosphate-buffered saline. The concentration was determined by measuring the  $A_{280}$  and checked by SDS-PAGE. 43 mg of the HuVHTA-YGD antibody and 20 mg of the chimeric antibody has been sent to USAMRIID.

### **3.1.12 Experiments at USAMRIID**

In *in vitro* virus neutralisation experiments performed at USAMRIID the 2nd generation HuVHTA-YGD antibody was equivalent to the first reshaped antibody. The original murine antibody was effective to approximately 10-fold lower concentration than the reshaped versions and neutralised more completely at high concentrations. In some experiments the reshaped antibody showed a greater requirement for complement. The ELISA results obtained at Scotgen were confirmed in that the HuVHTA-YGD gave higher readings than the first reshaped and the murine antibodies. In a competition ELISA the murine antibody competed effectively against the reshaped antibody, but in a reciprocal experiment the reshaped showed incomplete blocking. The reshaped antibody was blocked only by mouse antibodies of similar specificity or by polyclonal anti-junin antibody, indicating specificity of binding. The reshaped antibodies demonstrated virus-specific complement-dependent cytolytic activity, but required higher concentrations than the murine antibody (personal communication, A Schmaljohn).

## **3.2 ANTI-VACCINIA ANTIBODY**

### **3.2.1 Status at contract mid-term**

At the mid-term point of the contract, the VH and VK genes for the anti-vaccinia antibody (VVI) had been cloned and the sequencing of these was in progress. After verification of the cell line, VVI10F5-4-1, by antibody isotyping and anti-vaccinia ELISA of conditioned medium, RNA was prepared and cDNA synthesised. The VH and VK genes were amplified by PCR and cloned into M13mp18 and M13mp19 for DNA sequence determination.

### **3.2.2 Sequencing of VVI VH**

The VVI VH gene was found to contain two internal *Pst*I sites in addition to the one introduced at the 5' end of the gene by the oligonucleotide used for amplification. Therefore the DNA was cloned into M13 as both *Pst*I fragments and *Pst*I to *Bst*II fragments. The sequence of the VVI VH gene was completed with the *Pst*I-*Bst*II fragment, which was cloned into both M13mp18 and M13mp19 to allow sequencing in both directions. The sequencing strategy is shown in figure 9. Confirmatory sequence information was obtained from a second cDNA synthesis and PCR amplification. From over 2000 base pairs sequenced, there were just two single base changes in two separate clones, at positions 138 and 257. These changes were thus aberrations of the PCR process.

The VVI VH DNA sequence and its protein product are shown in figure 10 with the complementarity determining regions (CDRs) outlined. The first eight and the last eleven amino acids are determined by the oligonucleotide primers used and are not

necessarily those in the mouse antibody. VVI VH is most closely related to Kabat subgroup IIB (12). The alignment of VVI VH with the consensus sequence of subgroup IIB shows that the framework regions are a good match (figure 11). Of the four residues that differ only one, namely alanine rather than valine at Kabat position 12, is unusual and this change is far removed from the first CDR. Residue number 71 is considered to be important in supporting the conformation of the CDR (13). As this is alanine in VVI VH, rather than the valine which is normal for the subgroup, it was decided to alter this residue to alanine in the human framework during reshaping.

### **3.2.3 Sequence of VVI VK**

Amplified VVI VK DNA was cloned into M13mp19 as PvuII to BglII fragments for sequencing. DNA from a second cDNA synthesis and PCR amplification was cloned into M13mp18 to obtain confirmatory sequence from the opposite strand. The sequencing strategy is shown in figure 12. The sequence determined from the second cloning was identical to that from the first, apart from the duplication of 5 nucleotides at the 3' end of the gene within the region of the VK1FOR primer. This was probably due to the primer slipping during PCR amplification.

Figure 13 shows the DNA sequence and the protein product with the CDRs outlined. The first eight and last seven amino acids are dictated by the primers used. The sequence of VVI VK is most closely related to Kabat subgroup VI (12). Figure 14 shows the alignment of VVI VK with the consensus sequence for subgroup VI. The framework regions of VVI VK differ from the consensus only at Kabat position 100, which is serine rather than alanine. This change is also found in two other members of the subgroup. No alterations to the standard framework were proposed for the reshaped VVI VK.

### **3.2.4 Transplanting CDRs into human frameworks**

The human frameworks used for VVI VH and VK are derived from NEWM and REI respectively. Synthetic oligonucleotides were made which consisted of the VVI CDR sequences with 12 residues from the appropriate human framework sequence added at either end. The VVI CDR sequences were transferred by oligonucleotide-directed mutagenesis of the human VH and VK genes in M13 (VHPCR1 and VKPCR2 respectively) or by PCR. The sequences of the reshaped VVI VH and VK genes, with the mutagenic oligonucleotides underlined, are shown in figures 15 and 16. For VVI VH, the oligonucleotide for CDR1 has been extended at the 5' end to include the change from serine, which is a very unusual residue in NEWM, to tyrosine at Kabat position 27. The oligonucleotide for CDR2 has been extended at the 3' end to encompass the change at Kabat position 71 from valine to the alanine found in the original VVI VH.

On completion of the mutagenesis, the reshaped VVIHuVH and VVIHuVK genes were completely sequenced to ensure that no spurious changes had occurred.

### **3.2.5 Cloning of reshaped VVI VH and VK into expression vectors**

The reshaped VVIHuVH was cloned into the vector pSVgpt as a HindIII to BamHI fragment. The human IgG1 constant region was added separately as a BamHI fragment (figure 1). In the same way the reshaped VVIHuVK gene was cloned into the vector pSVhyg (figure 2). In this plasmid the hygromycin (hyg) resistance gene is substituted for the gpt gene and the human kappa constant region is already included.

### **3.2.6 Transfections into myeloma cells and selection of antibody producing cell lines**

The VVIHuVH expression plasmid (pSVgptVVIHuVH.HuIgG1) and a 2-fold excess of the VVIHuVK expression plasmid (pSVhygVVIHuVK.HuCK) were cut with PvuI to linearise and cotransfected into approximately  $10^7$  YB2/0 rat myeloma cells by electroporation. 24 to 48 hours later the cells were distributed into 24 well plates in medium containing xanthine and mycophenolic acid and incubated for a further 14 days. In the first transfection experiment only 3/24 wells contained colonies. Medium from these tested positive in an ELISA for human IgG and in a preliminary assay showed binding to inactivated vaccinia virus. A repeat of the transfection experiment resulted in 24/24 wells containing antibody producing cell clones.

### **3.2.7 Purification of antibody**

6 transfectants were expanded and reshaped antibody was purified from 500 ml of conditioned medium by protein A affinity chromatography. Murine antibody was similarly prepared from the original mouse hybridoma, VVI-10F5-4-1. The antibody concentrations were determined by measuring the  $A_{280}$  and checked by SDS-PAGE.

### **3.2.8 ELISA of binding to vaccinia virus**

The purified antibodies were tested by ELISA for binding to inactivated vaccinia virus. Although the initial experiment showed little difference between them, repeated determinations indicated that binding by the reshaped antibody is reduced approximately 10-fold compared to the original murine antibody (figure 17).

### **3.2.9 Antibody competition assay**

A competition ELISA was set up in which biotinylated murine antibody was the first antibody and was competed by increasing amounts of either the murine antibody itself, the reshaped antibody or an irrelevant control antibody. Figure 18 shows that there was only slight competition by the reshaped compared to the murine antibody, but that there was no competition at all by the control antibody. Therefore the reshaped antibody is binding to the same epitope as the original murine antibody, but the reduced affinity is confirmed.

### **3.2.10 Immunoblotting**

Western blots of non-reduced vaccinia antigen preparation and reshaped and murine antibodies are shown in figure 19. Samples of the VVI antibodies were also loaded onto the gel as controls. Detection was by biotin-conjugated anti-mouse or anti-human IgG followed by avidin-alkaline phosphatase. Colour was developed with bromochloroindolyl phosphate/nitro blue tetrazolium substrate. A vaccinia band of approximately 26 kD was detected by the murine antibody as expected. The control antibody samples on the gel were detected appropriately by the anti-mouse and anti-human reagents, indicating successful functioning of the reagents on the membrane. The reshaped antibody seemed to contain an excess of free kappa chain, seen as a band of approximately 25 kD in the antibody track of panel A, but no band was seen in the vaccinia track.

### **3.2.11 Construction of chimeric VH and VK genes**

The chimeric VK gene was constructed by inserting VVIMuVK (from PCR amplification of cDNA) cut with PvuII and BglII into M13VKPCR2 cut with PvuII



and BclI. The latter site was removed from the reshaped constructs as it causes the inclusion of an unusual amino acid in the antibody chain.

Because there are two internal PstI sites, VVIMuVH can not simply be inserted into M13VHPCR1 in the same way. Instead the construction of the chimeric heavy chain was by "sticky-feet"-directed mutagenesis (section 2.9). An M13 clone containing the desired substitution was obtained, but was found to have a spurious deletion of a GC base pair at the 5' end. This was corrected by a further mutagenesis.

The chimeric variable regions were cloned into the expression vectors pSVhyg and pSVgpt as for the reshaped. Appropriate constant regions were added as before.

### 3.2.12 Production of chimeric antibody

The chimeric VVIMuVH and VVIMuVK expression vectors (pSVgptVVIMuVH.HuIgG1 and pSVhygVVIMuVK.HuCK) were cotransfected into YB2/0 cells. Antibody-producing cell lines were selected by ELISA and expanded. Chimeric antibody was purified from conditioned medium by protein A affinity chromatography as before.

### 3.2.13 Analysis of binding of chimeric antibody to vaccinia by ELISA and Immunoblot

An ELISA of binding to inactivated vaccinia by the reshaped, the chimeric and the murine VVI antibodies is shown in figure 20. The chimeric antibody showed slightly reduced binding compared to the murine antibody, but the difference may be partly due to the different reporter antibodies used. The binding of the reshaped antibody was, as expected, less than the chimeric antibody.

The Western blot was repeated with the murine, chimeric and reshaped antibodies. Samples of the antibodies were loaded on the gel as controls as before. Figure 21 shows that the murine and the chimeric antibodies both lit up a band of 26 kD in the vaccinia track, but no band was seen with the reshaped antibody.

### 3.2.14 Mix and Match antibodies

Hybrid chimeric/reshaped antibodies were produced as for the anti-junin project, to determine whether the reduced binding of the reshaped antibody was due to the heavy or the light chain variable region. Therefore the chimeric heavy chain (VVIMuVH) and the reshaped light chain (VVIHuVK) expression vectors and the reshaped heavy chain (VVIHuVH) and chimeric light chain (VVIMuVK) expression vectors were cotransfected into YB2/0 cells. Antibody-producing cell clones were selected and expanded and "mix and match" antibodies purified from conditioned medium as before.

The antibody preparations were found to contain some bovine antibody (from the serum in the medium) so the concentrations were adjusted from the results of an ELISA for human IgG before use. An ELISA of binding of the chimeric, reshaped and "mix and match" antibodies is shown in figure 22. The MuVH/HuVK antibody performed as well as the chimeric MuVH/MuVK antibody, with the HuVH/MuVK antibody falling between these and the reshaped antibody. In a Western blot of vaccinia antigen with these antibodies, only the chimeric and the MuVH/HuVK antibodies lit up the expected 26 kD band (data not shown). These results indicate that the reduced binding of the reshaped VVI antibody is mainly due to the reshaped heavy chain.

### **3.2.15 Large-scale antibody production**

15 litres of conditioned medium from the reshaped VVI antibody producing lines was collected and the cells removed. This was then concentrated by cross-flow filtration through a 100 kD filter. The antibody was purified by protein A affinity chromatography and antibody containing fractions pooled and dialysed against PBS. The concentration was determined by measuring the  $A_{280}$  and checked by SDS-PAGE (figure 23). 25 mg of the reshaped VVI antibody together with a small sample of the chimeric antibody has been shipped to USAMRIID.

### **3.2.16 Results from USAMRIID**

In assays undertaken at USAMRIID the reshaped VVI antibody shows virus neutralising activity, but is approximately 50-fold less effective than the murine progenitor. In this assay the chimeric performs as well as the murine antibody, indicating that the human constant region is not adversely affecting the results. Competition ELISAs confirmed the results obtained at Scotgen. The reshaped antibody did not completely inhibit binding by labelled murine antibody (personal communication, A Schmaljohn).

### **3.2.17 Development of a "2nd generation" reshaped antibody**

A comparison of the sequences of VH and VK of the reshaped and murine VVI antibodies is shown in figure 24. The results of the "mix and match" antibody experiments indicated that the structure of the reshaped heavy chain variable region was most likely causing the reduced binding efficiency. Our attention was drawn to the double proline residue found at positions 40 and 41 in NEWM, which was not present in the original murine VVI or other mouse antibodies of the same subgroup (12). As proline residues cause sharp turns in protein chains, it was decided to replace the double pro with arg pro from the murine VVI antibody.

The required alteration was made to the reshaped VVI VH in M13 by PCR (section 2.8) and the new version cloned into the expression vector as before. The sequence of the variable region was checked in this plasmid. This construct has now been cotransfected with the reshaped VK plasmid into YB2/O cells. When antibody-producing cell clones are obtained, 2nd generation reshaped antibody will be isolated and tested as before.

## **4. DISCUSSION**

During the second half of this contract, the production of reshaped (CDR-grafted) antibodies from the two murine monoclonal antibody-producing cell lines supplied by USAMRIID was completed. Chimeric antibodies consisting of murine variable regions linked to human constant regions have also been produced. These chimeric antibodies generally bind as well as their murine counterparts and are considered to be important controls for the reshaped antibodies; potential artefacts are avoided by using the same detection reagents in immunoassays and for neutralisation assays the same effector functions are provided by the same antibody constant regions.

The first reshaped anti-junin antibody made performed as well as the murine antibody in an ELISA using inactivated junin virus. However, preliminary data on virus neutralisation indicated that the reshaped antibody was 10-fold less effective than the murine progenitor. Therefore, the chimeric, "mix and match" and "2nd generation" reshaped antibodies were constructed. The chimeric antibody, the first

reshaped antibody and the murine antibody were all equivalent in the ELISA. The "2nd generation reshaped" HuVHTA-YGD and hybrid antibodies containing this heavy chain showed at least 4-fold better binding by ELISA. These results were confirmed at USAMRIID, but *in vitro* virus neutralisation assays showed no difference between the two reshaped antibodies, both being about 10-fold less effective than the murine antibody. The neutralisation results were confirmed by a competition ELISA, in which the reshaped antibody showed incomplete blocking of the murine antibody, although there was evidence that specificity had been retained.

The chimeric VVI antibody performed as well as the original murine monoclonal in ELISAs of binding to inactivated vaccinia antigen and in Western blot assays lit up the same band of approximately 26 kD, indicating that the correct antibody gene sequences had been cloned. The reshaped antibody showed 10-fold reduced binding by ELISA and no activity in a Western blot. In virus neutralisation assays done at USAMRIID, the reshaped antibody does neutralise, but not as well as the murine progenitor, reflecting the ELISA results. In this assay the chimeric performs as well as the murine antibody, confirming that the reduced affinity is due to the antibody variable region. Competition ELISAs, done both at Scotgen and at USAMRIID, showed that the reshaped antibody could not compete as well as the murine antibody with itself. The "mix and match" antibody experiments indicated that the reduction in binding efficiency was mainly due to the reshaped heavy chain.

It is now becoming obvious, both from our own personal experience and from published papers from other groups (14, 15, 16, 17, 18) that reshaping or *humanising* antibodies by simple CDR grafting is not necessarily effective. Certain residues in the frameworks of the antibody variable regions are important in the determination of the functional conformation of the CDRs and certain critical residues from the original mouse antibody may need to be substituted into the human framework used. It may also be necessary to consider the alternative definition by Chothia and Lesk (19) of the CDRs as loop regions on the surface of the antibody molecule compared to the hypervariable regions defined by Kabat (12). The knowledge of changes that are likely to be important in the restoration of binding efficiency is being increased all the time and techniques of 3-dimensional antibody modelling are also progressing. However, all proposed modifications must be tested in practice. Scotgen have based most of their reshaping on one set of human frameworks, NEWM for the heavy chain and REI for the light chain, for which an extensive database has already been acquired so that the minimum number of murine residues are included in the final reshaped antibody. This contrasts with the approach of others (15, 16) where the most "homologous" human frameworks are chosen and many murine residues transferred to the reshaped antibody. This raises the question of how many mouse residues can be substituted into the human framework and it remain a "human" antibody? Apart from providing a more "human" antibody, another advantage of Scotgen's approach is that an antibody based on NEWM and REI has already been used in patients with no ill effect.

In some cases, alterations in framework residues cause dramatic effects on binding efficiency, as with an anti-RSV antibody where residues FCNS in the heavy chain immediately before CDR3 were substituted for YCAR to restore binding (14). However, Kettleborough *et al* (17) tried a variety of modifications, most of which had only a small, but cumulative effect. In the reshaped YGD antibody, substitution of residues at positions 70 and 71 with "TA" as in the murine antibody (see figure 5) resulted in an increase in binding measured by ELISA. This

demonstrates that it is possible to achieve equivalent binding to the mouse antibody with a minimum number of "mouse" substitutions in the human framework. The same principle should be true for vaccinia antibody such that the subtle substitution at position 40 should improve binding.

From the data obtained, it appears that the binding efficiency of the reshaped anti-vaccinia antibody correlates with the extent of vaccinia virus neutralisation, but in each case is 10 to 50-fold reduced compared to the progenitor mouse antibody. Thus, post-contract work has concentrated on modifications to the reshaped heavy chain designed to improve the binding and neutralising activity of the reshaped antibody. For the reshaped anti-junin antibody no such correlation between binding and neutralising ability is apparent, such that the "2nd generation" HuVHTA-YGD antibody exhibits greater binding efficiency than the murine antibody, but lower efficiency of neutralisation. While this might reflect an influence of the mouse versus human constant regions of the two antibodies, it is more likely to be due to an avidity component of binding to intact virus which is lost on reshaping. Both the murine and reshaped antibodies may bind to the disaggregated junin virus antigen in the ELISA assay to a similar extent, but the murine antibody may show greater bivalent association with neighbouring antigen molecules in intact virus. This could reflect differences in the flexibility of the mouse IgG2b versus human IgG1 heavy chain hinge region in the respective antibodies, or alternatively differences in the orientation of antigen-antibody binding between the antibodies.

In our experience the in vitro differences in affinity between reshaped and murine antibodies are not indicative of altered in vivo properties and the data from in vivo studies at USAMRIID is awaited.

## **5. CONCLUSIONS**

### **5.1 Implications**

- (1) It is possible to produce reshaped or humanised antibodies which recognise and neutralise viruses.
- (2) Commonly a loss of binding and/or neutralisation efficiency may occur during reshaping.
- (3) However, this can be rectified by minimal modifications to the human framework regions. Such modifications are too small to be likely to induce an immune response in man to these antibodies.

### **5.2 Recommendations**

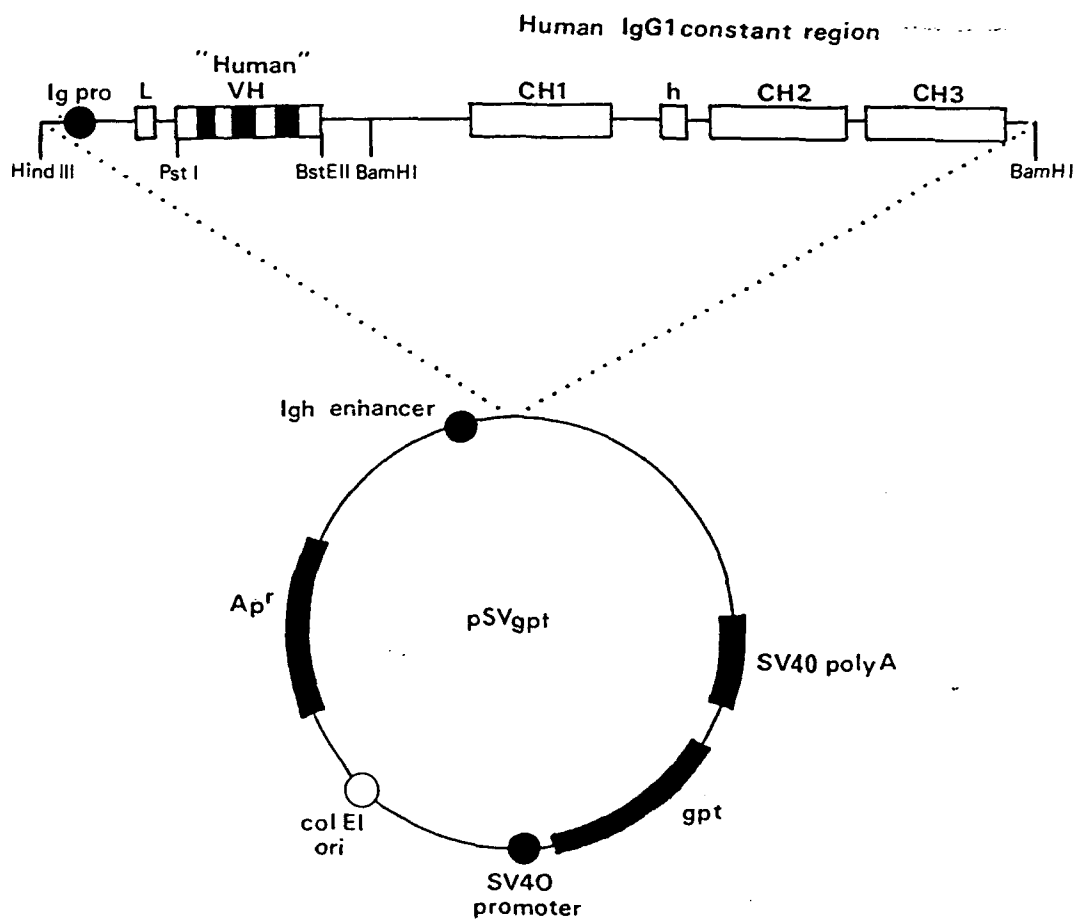
- (1) Further subtle modifications be made to the antibody variable regions to increase neutralisation. Candidate modifications have already been proposed for anti-junin and anti-vaccinia antibodies.
- (2) This process (1) be assisted by improved 3-dimensional modelling of reshaped antibodies.

- (3) The effect of the substitution of different human constant regions on virus neutralisation by the anti-junin antibody be investigated.
- (4) The effectiveness of these antibodies in animal models be investigated.
- (5) The successful reshaping of murine anti-junin and anti-vaccinia antibodies demonstrated here indicates that this technology should now be applied widely for the humanisation of other antibodies against infectious diseases.

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**FIGURE 1**

Components and organisation of immunoglobulin heavy chain mammalian expression vector (not to scale).



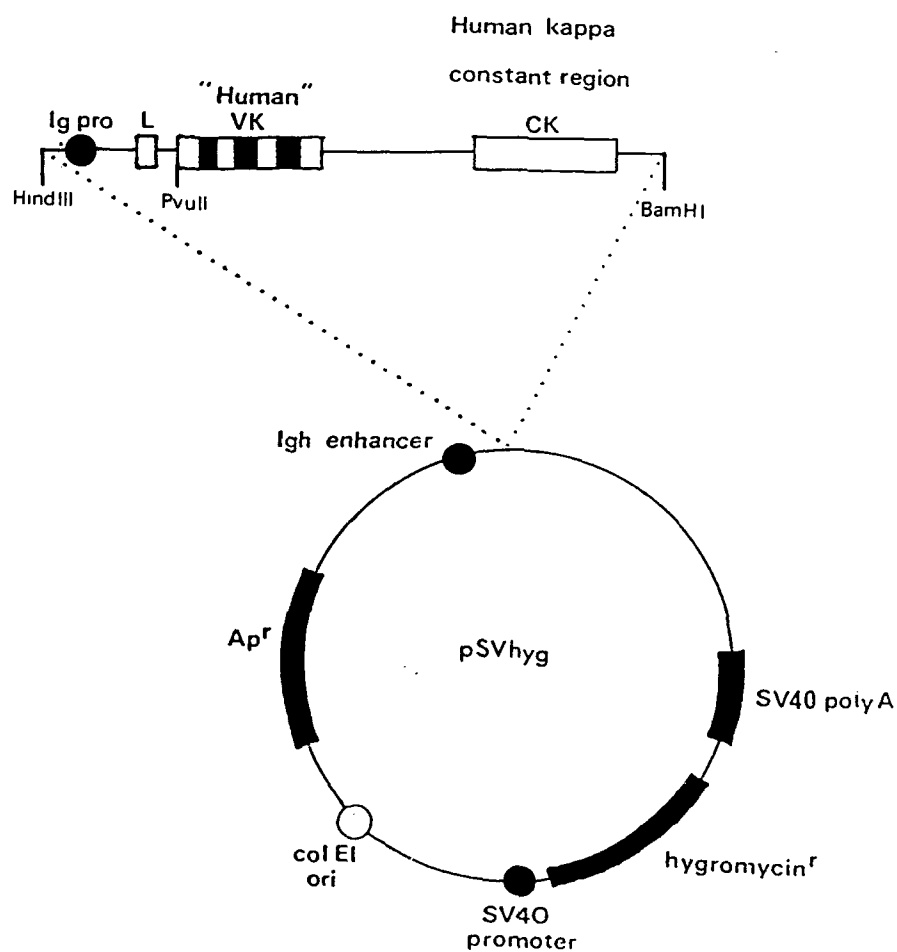


FIGURE 2

Components and organisation of immunoglobulin light chain mammalian expression vector (not to scale).

a

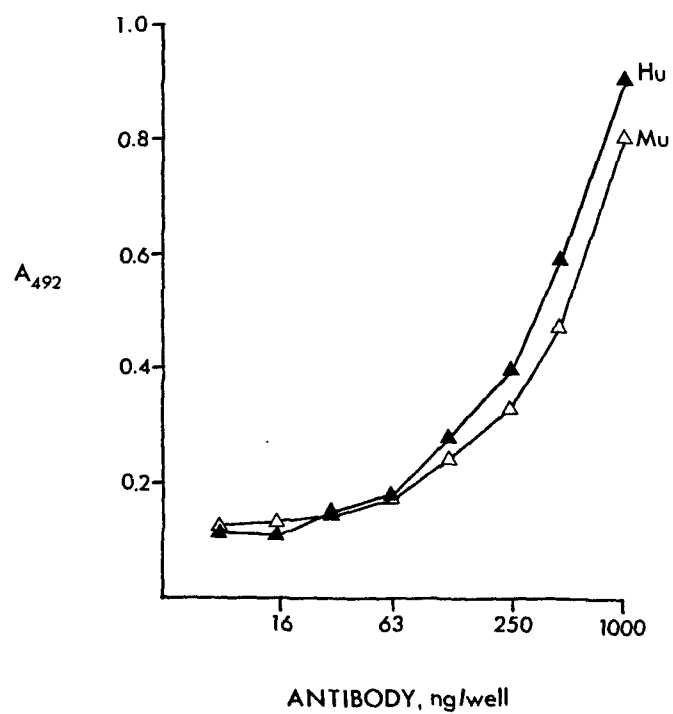
	10v	20v	30v	40v	50v
YGDVH	QVQLQQSGAELARPGTSVKLSCKASGYTF	IN	YWMQWIKQRPQG	LEWIGAV	
	QVQLQQ:GAEL.:PG:SVKLSCKASGYTF	I:YWM:W:KQRPQG	LEWIG:		
MUVHIIB	QVQLQQPGAELVKPGASVKLSCKASGYTF	SYWMH	WVKQRPQG	LEWIGRI	
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
YGDVH	YPGDGDTFRFSQKFKG	KATLTADKSSSTAYMQLSSL	SS	SEDSAVYFCAR	RVY
	P..G:T:::KFK:	KATLT.DKSSSTAYMQLSSL:	SEDSAVY:CA	R	Y
MUVHIIB	DPNSGGTKYNEKFKS	KATLTVDKSSSTAYMQLSSL	TS	SEDSAVYYCA--	RVY
	60^	70^	80^	90^	100^
	110v	120v			
YGDVH	YGSNY-IYALDYWGQGT	TVTVSS			
	YGS: Y:DYWGQGT:	TVSS			
MUVHIIB	YGSSSGYVYFDYWGQGT	TLTVSS			
	110^	120^			

b

	10v	20v	30v	40v	50v
YGDVK	DIQLTQSPKFMSTSIGDRVSITC	KASQNVGSAVA	WYQQKPGQSPKLLIY	SA	
	DIQ:TQSP. :S:S:GDRV:ITC:ASQ:::	..WYQQKPG :PKLLIY	A		
MUVKV	DIQMTQSPSSLSASLGDRVTITC	RASODISNYLN	WYQQKPGGTPKLLIY	VA	
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
YGDVK	SNRYTGVPDRFIGSESGTDFTLTISNMQ	SEDLADYFC	QYSSYPLAF	GAGT	
	S. :GVP.RF GS.SGTD::LTIS::	ED:A.YFCQQ :S P :FG:GT			
MUVKV	SRLHS	GVP	SRFSGSGTDYSLTIS	LEQEDIATYFC	QGN
	60^	70^	80^	90^	100^
YGDVK	KLEI				
	KLEI				
MUVKV	KLEIK				

FIGURE 3

Comparison of amino acid sequences of (a) YGDVH with a consensus sequence of mouse VH subgroup IIb and (b) YGDVK with a consensus sequence of mouse VK subgroup V.



**FIGURE 4**

ELISA showing binding of murine YGD (Mu) and humanised YGD (Hu) antibodies to a crude junin virus preparation.

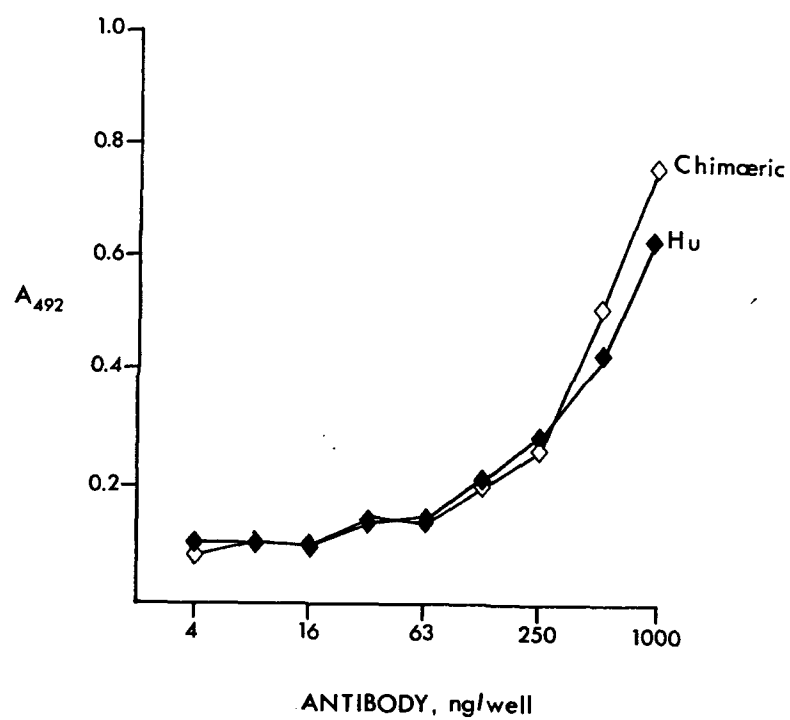
	10v	20v	30v	40v	50v
YGDVH	QVQLQQSGAELARPGTSVKLSCKASGYTF	NYWMQWIKORPGGLEWIGAV			
	QVQLQ:SG:.L.RP: ::.L:C..SGYTF:	NYWMQW::Q.PG:GLEWIGAV			
YGDHUVH	QVQLQESGPGGLVRPSQTLSTCTVSGYTF	NYWMQWVRQPPGRGLEWIGAV			
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
YGDVH	YPGDGDTFRFSQKFKGKATLTADKSSSTAYMQLSSLSEDSAVYFCARRRVY				
	YPGDGDTFRFSQKFKG:.T:.D.S.: ::LSS:::D:AVY:CARRRVY				
YGDHUVH	YPGDGDTFRFSQKFKGRVTMLVDTSKNQFSLRLSSVTAADTAVYYCARRRVY				
	60^	70^	80^	90^	100^
	110v	120v			
YGDVH	YGSNYIYALDYWGQGT	TVTVSS			
	YGSNYIYALDYWGQGT	TVTVSS			
YGDHUVH	YGSNYIYALDYWGQGT	TVTVSS			
	110^	120^			

	10v	20v	30v	40v	50v
YGDVK	DIQLTQSPKFMSTSIGDRVSITCKASQNVGSAVAWYQOKPGQSPKLLIYSA				
	DIQLTQSP. :S:S:GDRV:ITCKASQNVGSAVAWYQOKPG::PKLLIYSA				
YGDHUVK	DIQLTQSPSSLSASVGDRVITITCKASQNVGSAVAWYQOKPGKAPKLLIYSA				
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
YGDVK	SNRYTGVPDRFIGSESGTDFTLTISNMQSEDLADYFCQYSSYPLAFGAGT				
	SNRYTGVP.RF GS.SGTDFT:TIS::Q:ED:A.Y:CQYSSYPLAFG.GT				
YGDHUVK	SNRYTGVPSPRFSGSGSGTDFTFITISLQPEDATYYCQYSSYPLAFGQGT				
	60^	70^	80^	90^	100^
YGDVK	KLEI				
	K:EI				
YGDHUVK	KVEIK				

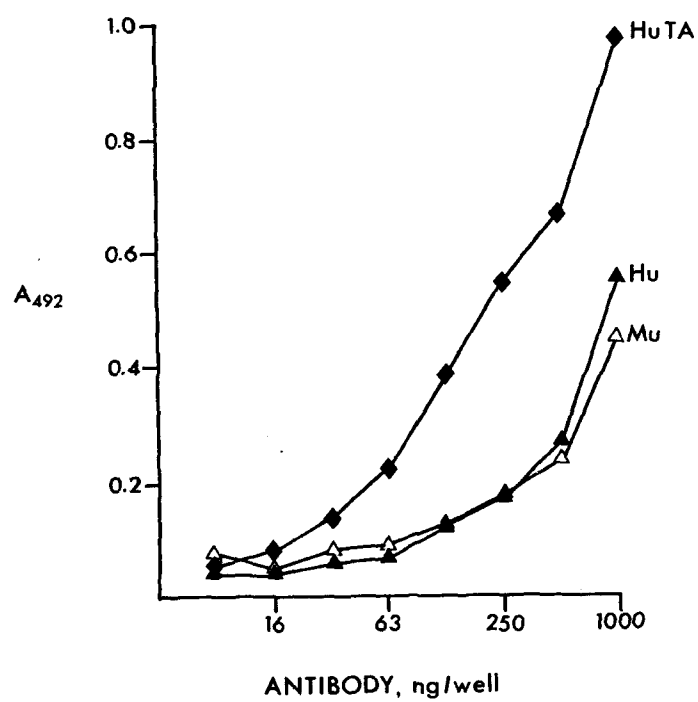
FIGURE 5

Alignment of amino acid sequences of murine and reshaped YGD VH and VK. CDRs are boxed and amino acids altered in the second generation VH are arrowed.



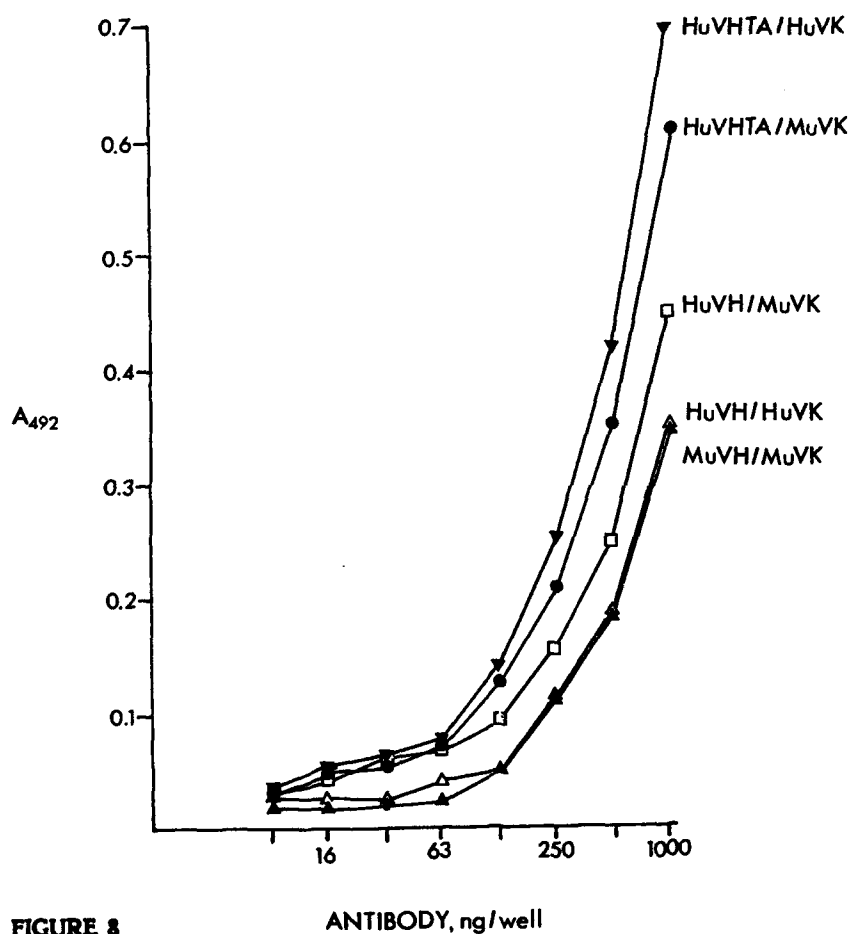
**FIGURE 6**

ELISA showing binding of chimeric YGD and humanised YGD (Hu) antibodies to a crude junin virus preparation.



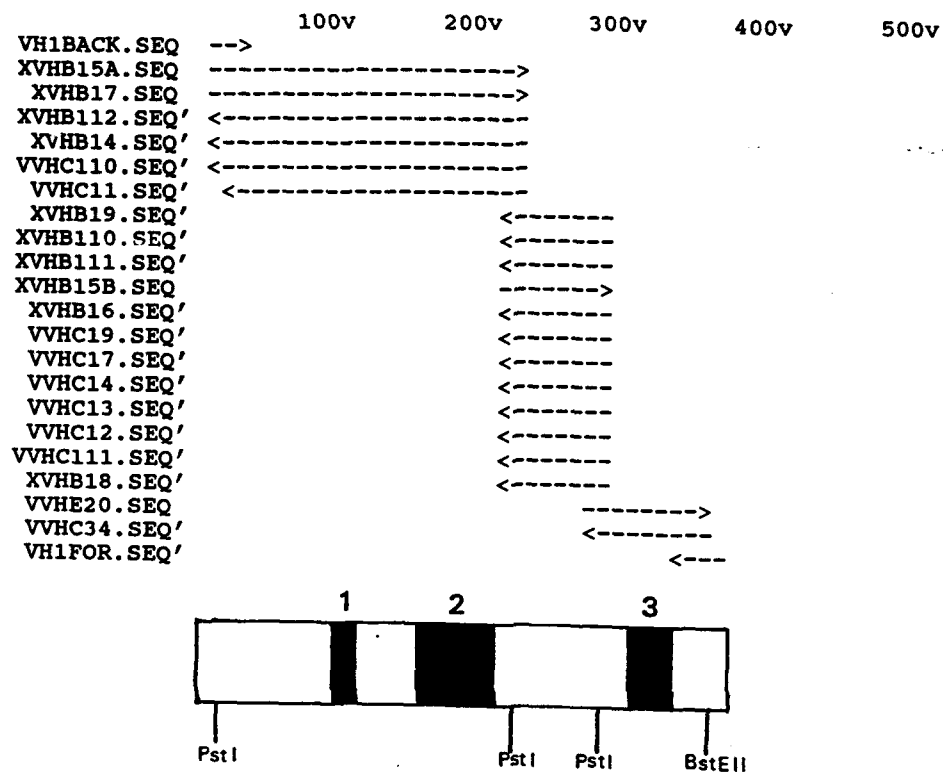
**FIGURE 7**

ELISA showing binding to a crude junin virus preparation by murine YGD (Mu), 1st generation reshaped YGD (Hu) and 2nd generation reshaped YGD (HuTA) antibodies.



**FIGURE 8**

ELISA showing binding to a crude junin virus preparation of murine YGD (MuVH/MuVK), 1st generation reshaped YGD (HuVH/HuVK), 2nd generation reshaped YGD (HuVHTA/HuVK) and two "mix and match" YGD (HuVHTA/MuVK and HuVH/MuVK) antibodies.



**FIGURE 9**

Sequencing strategy for M13 VVI VH clones.



P  
S  
T  
1

AGGTSNARCTGCAGGAGTCMGGGGCTGAACCTGGCAAAACCTGGGGCCTCA 50  
TCCASKTYGAGCTCTCAGNCCCGGACTTGACCGTTTTGGACCCCGGAGT

v ? l q e s g a e l a k p g a s

GTGAAGATGTCTCTCAAGGCTCTGGCTACACCTTTACTAGGTACTGGAT 100  
CACTTCTACAGGACGTTCCGAGACCGATGTGGAAATGATCCATGACCTA

v k m a c k a s g y t f t f y w s

GCACGGGTAAACAGAGCCCTGGACAGGCTCTGGAATGGATTGGATACA 150  
CGTGACCAATTTGTCTCCGAGCTGTCCAGACCTTACCTAACCTATGT

h v v k q r p q q g l e w i g y i

TTAATCCTAGCACTGGTTATACTGAGTACAATCAGAAGTTCAAGGACAAG 200  
AATTAGGATCGTGACCAATATGACTCATGTAGTCTTCAAGTTCCTGTTC

n p s t g y t e y n q k f k d k

P  
S  
T  
1

GCCACATTGACTCCAGACAAATCCTCCAGCACAGCCTACATGCAACTGAG 250  
CGGTGTAACTGACGCTCTGTTAGGAGTGGTGTGGATGTACGTTGACTC

a t l t a d k s s a t e y m q l s

P  
S  
T  
1

CAGCCTGACATCTGAGGACTCTGCACTCTATTACTGTGCAAGAGTGAAT 300  
GTGGACTGTAGACTCTGAGACGTGAGATAATGACAGTTCTTCACTGA

s l t s e d s a v y y c a r e d y

P  
S  
T  
1

ATAGGAACCTGGTACTTCGATGTCGGGCCAAGGGACACGGTCAACCGTC 350  
TATCCTTGACCATGAAGCTACAGCCCGGTTCCCTGGTCCAGTGGCAG

r n w y f d v v g q g t t v t v

TCCTCA 356  
AGGAGT

FIGURE 10

VVI VH DNA and protein sequence. The CDRs are boxed and the PCR primers underlined.

	10v	20v	30v	40v	50v
VVIVH	VXLQESGAELAKPGASVKMSCKASGYTFI	RYWMH	VVKQRPQG	GLEWIG	VI
	V.LQ::GAEL.KPGASVK:SCKASGYTFI	RYWMH	VVKQRPQG	GLEWIG	I
MUVHIIB	QVQLQQPGAELVKPGASVKLSCKASGYTFI	SYWMH	VVKQRPQG	GLEWIG	RI
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
VVIVH	NPSTGYTEYNQKFKDKATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	SDY-			
	:P::G T.YN:KFK.KATLT.DKSSSTAYMQLSSLTSEDSAVYYCAR	Y			
MUVHIIB	DPNSGGTKYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	YYG			
	60^	70^	80^	90^	100^
	110v				
VVIVH	---RNWYFDVWGQGTTLTVSS				
	.YFD WGQGTTLTVSS				
MUVHIIB	SSSGYVYFDYWGQGTTLTVSS				
	110^	120^			

**FIGURE 11**

Alignment of amino acid sequence of VVI VH with a consensus sequence of VH of mouse subgroup IIB.

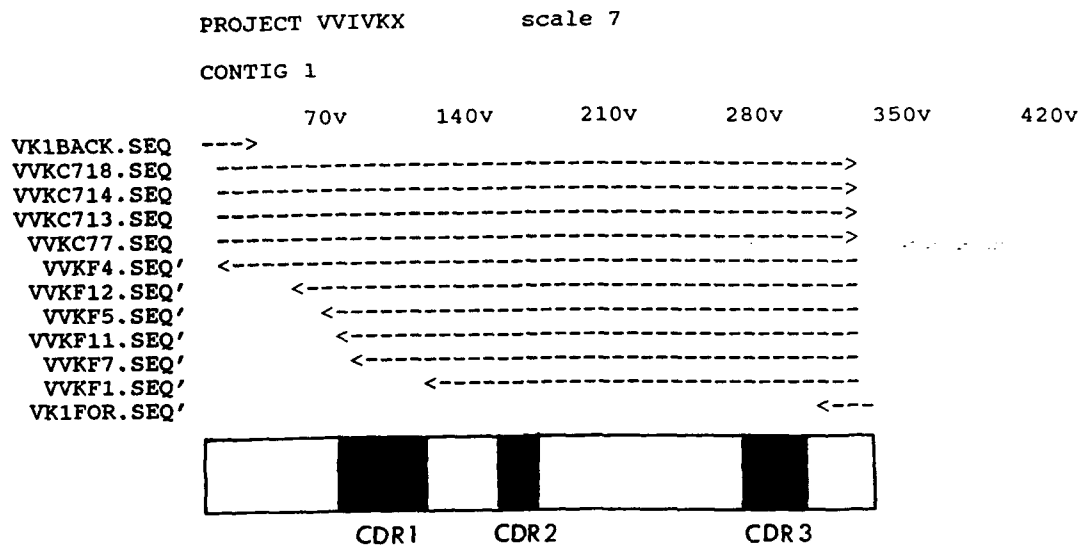


FIGURE 12

Sequencing strategy for VVI VK showing CDRs.

P  
 V  
 U  
 2

GACATTCAGCTGACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGA 50  
CTGTAAGTCGACTGGGTCAGAGGTCGTTAGTACAGACGTAGAGGTCCCCT  
 d i q l t q s p a i n s a s p g e  
 -----+-----+-----+-----+-----+

GAAGGTCACCATGACCTGCGTGCAGCTCAAGTGTAAATTACATGCAGT 100  
 CTTCCAGTGGTACTGGACGTCACGGTCGAGTTCACATTTAATGTACGTGA  
 k v t n t c s a s s s v n y m h w  
 -----+-----+-----+-----+-----+

GGTACCAGCAGAAGTCAGGCACCTCCCCAAAAGATGGATTTATGACACA 150  
 CCATGGTCGTCTTCAGTCCGTGGAGGGGTTTTCTACCTAAATACTGTGT  
 y q q k s g t s p k r w i y d t  
 -----+-----+-----+-----+-----+

TCCAAACTGGCTTCCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGG 200  
 AGGTTTGACCGAAGACCTCAGGGACGAGCGAAGTCACCGTCACCCAGACC  
 s k l a s g v p a r f s g s g s g  
 -----+-----+-----+-----+-----+

GACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCCA 250  
 CTGGAGAATGAGAGAGTGTTAGTCGTCTGTACCTCCGACTTCTACGACGGT  
 t s y s l t i s s n e e a e d a a t  
 -----+-----+-----+-----+-----+

CTTATTACTGCTCAGCAGTGGAGTCGTAACCCATTACGTTCCGGCTCGGGG 300  
 GAATAATGACCGTCGTACCTCAGCATTGGGTAAGTGCAGCCGAGCCCT  
 y y c g q w s r n p f t f g s g  
 -----+-----+-----+-----+-----+

B  
 G  
 L  
 2

ACCAAGCTGGAGATCTAAC 319  
TGGTTCGACCTCTAGATTG  
 t k l e i . r  
 -----+-----+-----+

FIGURE 13

DNA and protein sequence of VVI VK. PCR primers are underlined and CDRs are boxed.

		10v	20v	30v
VVIVK		DIQLTQSPAIMSASPGEKVTMTCSASSSVNY		
		:I LTQSPAIMSASPGEKVTMTCSASSSV:Y		
MUVKVI	MRVPAQLLGLLLLWLPGARGQIVLTQSPAIMSASPGEKVTMTCSASSSVSY			
		10^	20^	30^
		40v	50v	60v
VVIVK	MHWYQQKSGTSPKRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDA			
	MHWYQQKSGTSPKRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDA			
MUVKVI	MHWYQQKSGTSPKRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDA			
		60^	70^	80^
		90v	100v	90^
VVIVK	ATYYCQQWSRNPFTFGSGTKLEIX			
	ATYYCQQWS.NP:TFG:GTKLE:.			
MUVKVI	ATYYCQQWSSNPLTFGAGTKLELKR			
		110^	120^	

**FIGURE 14**

Alignment of amino acid sequence of VVI VK with a consensus sequence of mouse VK subgroup VI.

P  
S  
T  
I

CAGGTCCAACTGCAGGAGAGCGGTCCAGGTCTTGTGAGACCTAGCCAGAC  
 GTCCAGGTTGACGTCTCTCGCCAGGTCCAGAACACTCTGGATCGGTCTG  
 q v q l q e s g p g l v r p s q t  
 -----+-----+-----+-----+-----+

CCTGAGCCTGACCTGCACCGTGTCTGGCTACACCTTCAGCAGGTACTGGA  
 GGACTCGGACTGGACGTGGACAGACCGATGTGGAAGTCCTCCATGACCT  
 l s l t c t v s g y t f s r y w n  
 -----+-----+-----+-----+-----+

TGCAGTGGGTGAGACAGCCACCTGGACGAGGTCTTGTGAGTGGATTGGATAC  
 ACCTGACCCACTCTGTCTGGTGGACCTGCTCCAGAACTCAGCTAACCTATG  
 h v v r q p p g r g l e w i g y  
 -----+-----+-----+-----+-----+

ATTAACTCTAGCACTGGTTATCTGAGTACAATCAGAAGTTCAAGGACAG  
 TAATTAGGATCGTGACCAATATGACTCATGTTAGTCTTCAAGTCTCTGTG  
 i n p s t g y t e y n q k f k d  
 -----+-----+-----+-----+-----+

AGTGACAATGCTGGCAGACACCGCAAGAACCACTTCAGCCTGAGACTCA  
 TCACTGTTACGACCGTCTGTGGTCTTCTTGGTCAAGTCGGACTCTGAGT  
 v t m l a d t s k n q f s l r l s  
 -----+-----+-----+-----+-----+

GCAGCGTGACAGCGCGGACACCGCGGTCTATTATTGTGCAAGAGTGAC  
 CGTCGCACTGTGGCGGCTGTGGCGCCAGATAATAACAGCTTCCTCACTG  
 s v t a a d t a v y y c a r s d  
 -----+-----+-----+-----+-----+

B  
S  
T  
E

TATAGGAACCTGTAATCTGATGTCTGGGGCCAAAGGACCAAGGTCAACCGT  
 ATATCTTGAACATGAAGCTACAGACCCCGGTTCCTGCTGCTGCTGGCA  
 y r n w y f d v w g q g t t v t v  
 -----+-----+-----+-----+-----+

CTCCTCA  
 -----+ 357  
 GAGGAGT  
 s s  
 -----+

FIGURE 13

DNA and amino acid sequence of reshaped VVI HuVH. Mutagenic oligonucleotides are underlined and CDRs are boxed.

P  
 V  
 U  
 2

GACATCCAGCTGACCCAGAGCCCAAGCAGCCTGAGCGCCAGCGTGGGTGA 50  
 CTGTAGGTCGACTGGGTCTCGGGTTCGTGGACTCGCGGTCCACCCACT  
 d i q l t q s p s s l s a s v g d

CAGAGTGACCATCACCTGTAGTCCAGCTCAAGTGTAAATTACATGCAGT 100  
 GTCTCAGGTAGTGGACATCAGGTGAGTTCACATTAAATGACGTCA  
 r v t i t c s a s s v n y m h w

GGTACCAGCAGAAGCCAGGTAAGGCTCCAAAGCTGCTGATCTACACACA 150  
 CCATGGTGGTCTTCGGTCCATTCGGAGGTTTCGACGACTAGATCTGTGT  
 y q q k p g k a p k l l i y d t

TCCAACTGGCTTCTGCTGTGCCAAGCAGATTGAGCGGTAGCGGTAGCGG 200  
 AGGTTTGACCGAAGATCCACAGGTTGCTTAAGTCGCCATCGCCATCGCC  
 s k l a s p v p s r f a g s g s g

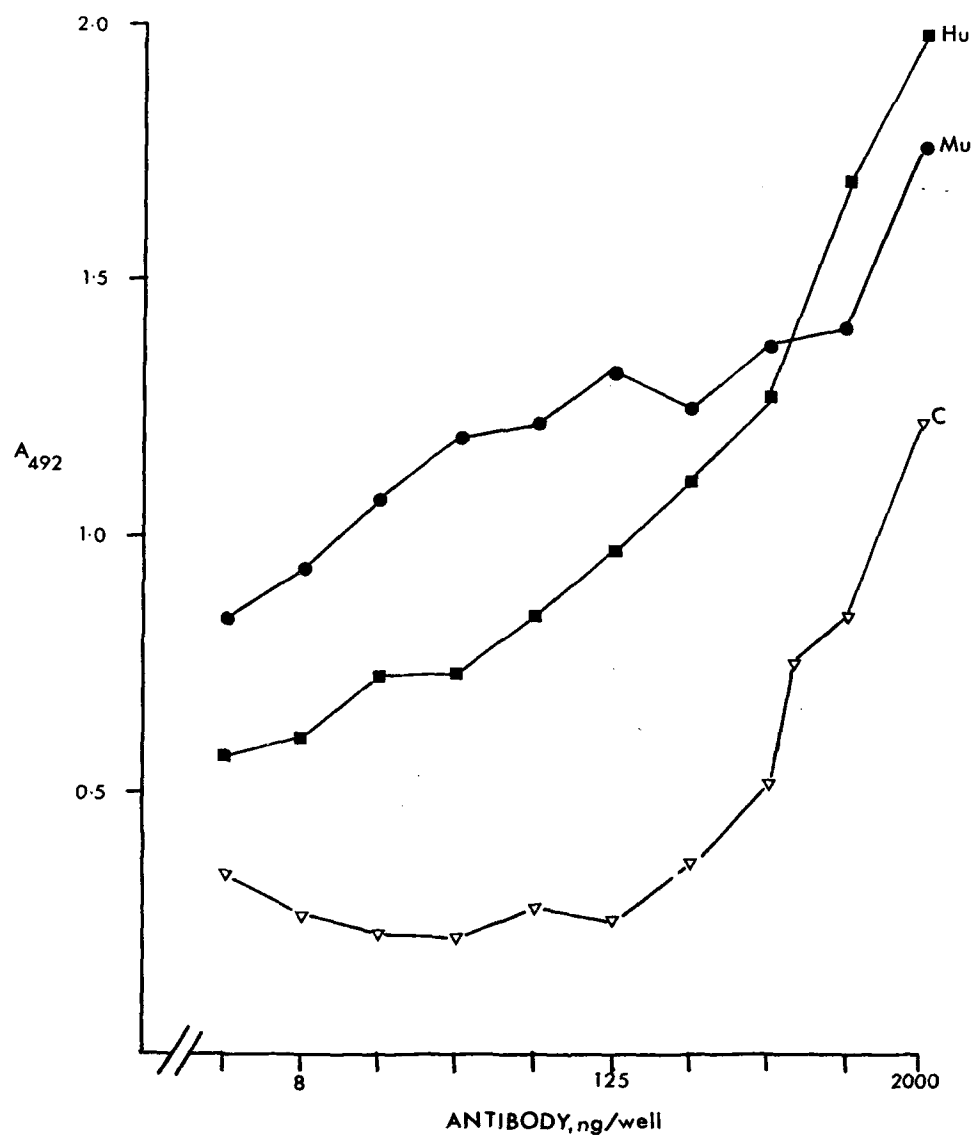
TACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCAGAGGACATCGCCA 250  
 ATGGCTGAAGTGAAGTGCTAGTCTGTCGGAGGTCGGTCTCCTGTAGCGGT  
 t d f t f t i s a l q p e d i a t

CCTACTACTGCAGCACTGGAGTGGTAAGCCATTACGTTCCGCCAAGGG 300  
 GGATGATGACGTCGTCACCTCAGCATTTGGGTAAGTGCAGCCGGTTCCC  
 y y c h q w s r n p f t f g q g

ACCAAGGTGGAAATC 315  
 TGGTTCCACCTTAG  
 t k v e i

FIGURE 16

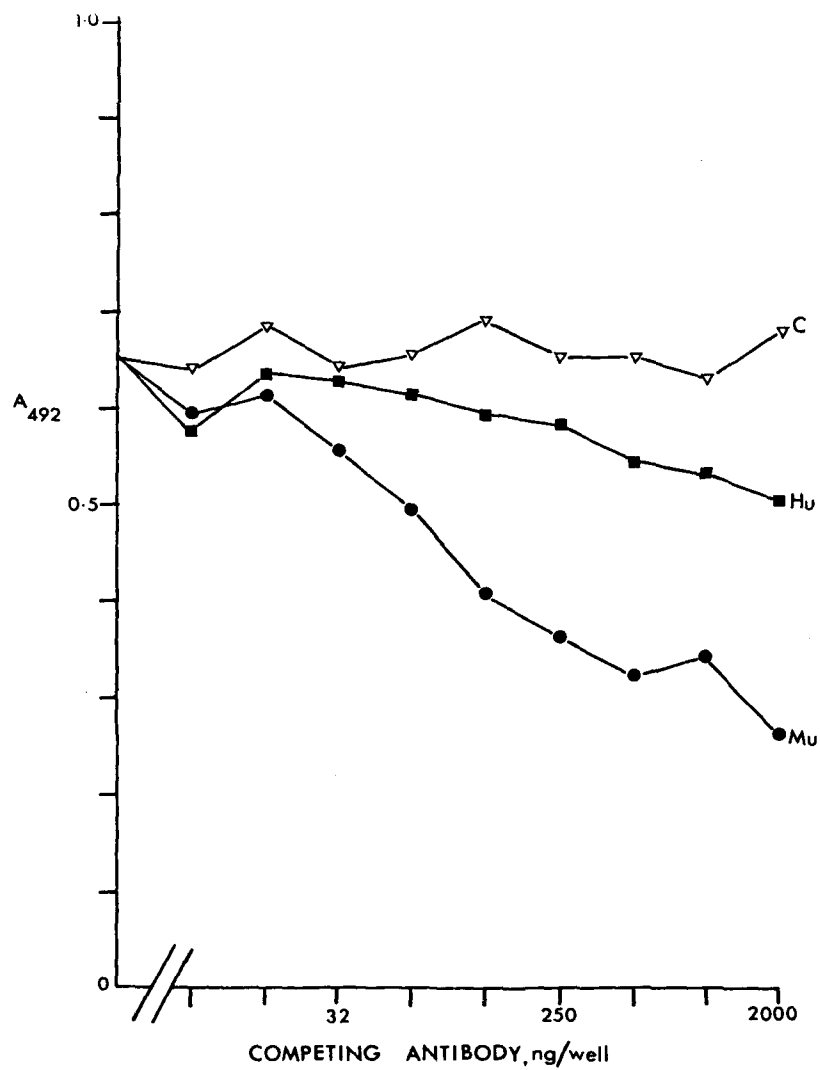
DNA and amino acid sequence of VVI HuVK. Mutagenic oligonucleotides are underlined and CDRs are boxed.



**FIGURE 17**

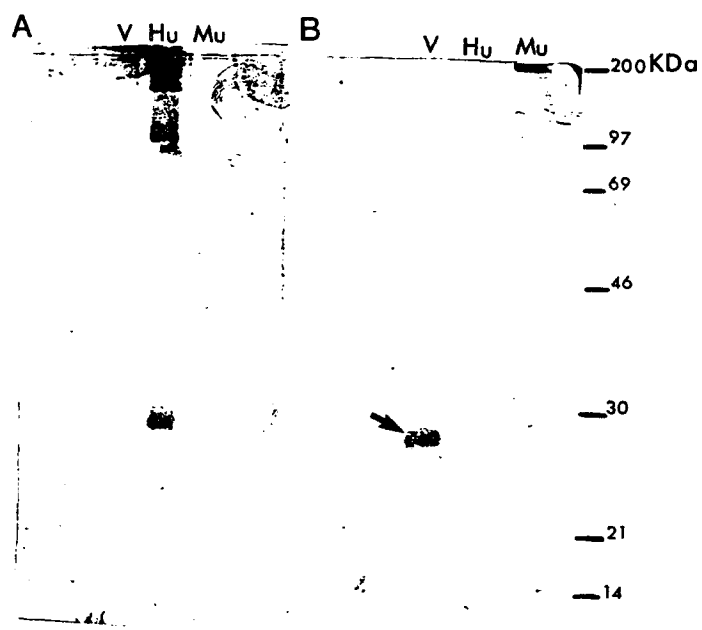
ELISA showing binding of reshaped VVI (Hu), murine VVI (Mu) and our irrelevant control antibody (C) to vaccinia antigen preparation.





**FIGURE 18**

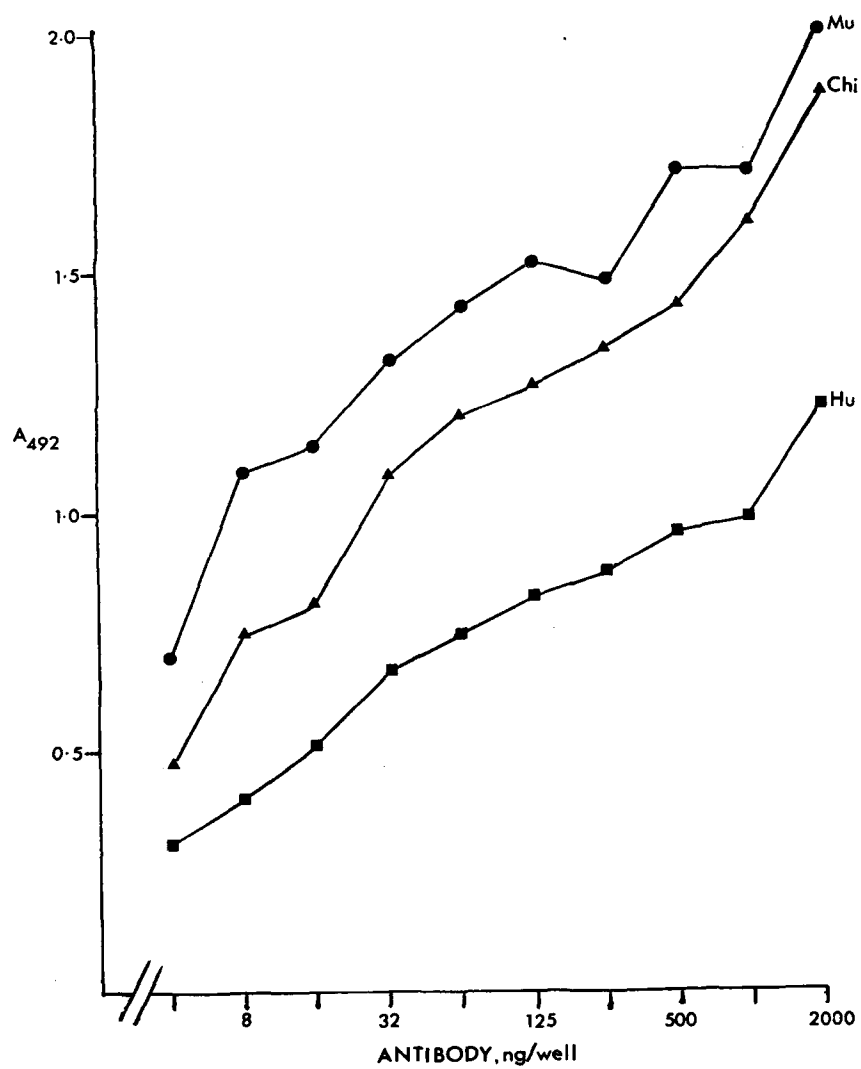
ELISA showing competition of binding of biotin-conjugated murine VVI antibody by unlabelled murine VVI (Mu), reshaped VVI (Hu) and a control antibody (c).



**FIGURE 19**

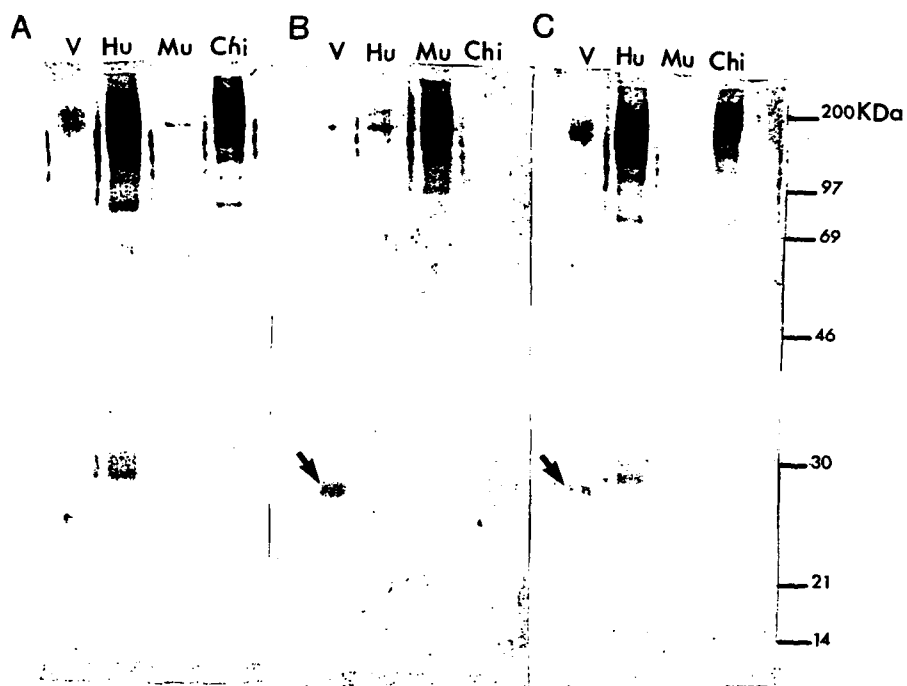
**A** Western blot of binding to non-reduced vaccinia antigen preparation by

- a. Reshaped VVI (Hu)
- b. Murine VVI (Mu)



**FIGURE 20**

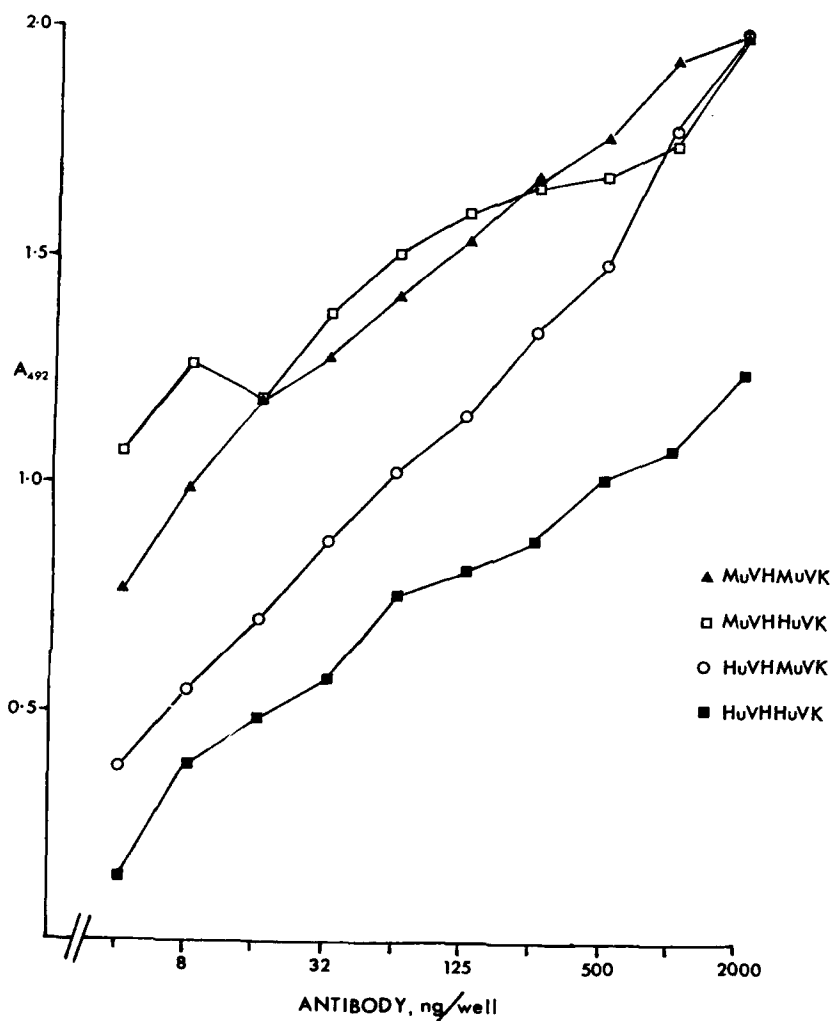
ELISA showing binding of reshaped VVI (Hu), murine VVI (Mu) and chimeric VVI (Chi) antibodies to vaccinia antigen preparation.



**FIGURE 21**

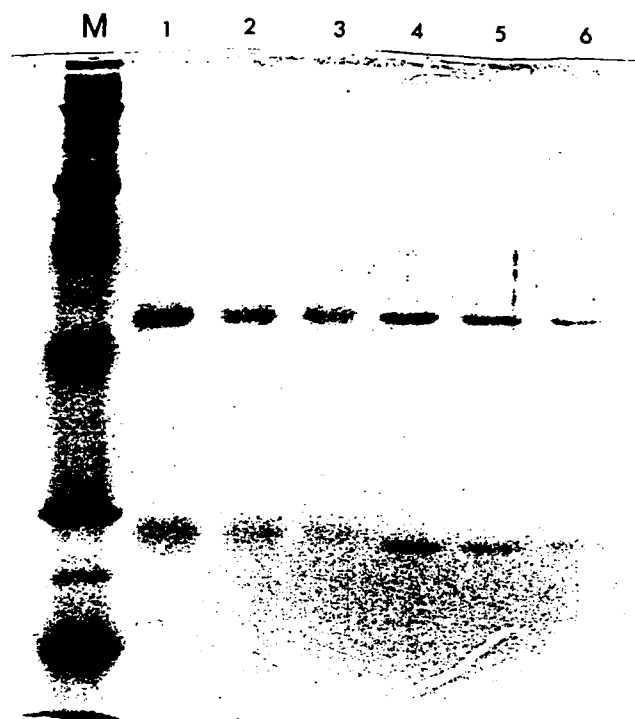
Western blot of binding to non-reduced vaccinia antigen preparation by:  
 (A) Reshaped VVI (Hu), (B) Murine VVI (Mu) and (C) Chimeric VVI (Chi)

V - vaccinia  
 Hu - reshaped VVI  
 Mu - murine VVI  
 Chi - chimeric VVI



**FIGURE 22**

ELISA showing binding to vaccinia antigen preparation of reshaped (HuVH.HuVK), chimeric (MuVH.MuVK) and mix and match VVI antibodies (MuVH.HuVK and HuVH.MuVK).



**FIGURE 23**

SDS-PAGE analysis of protein A purified reshaped VVI antibody. Lane 1, 2 ug control Ab, Lane 2, 1 ug control Ab, Lane 3, 0.5 ug control Ab, Lane 4, 2 ug reshaped VVI Ab, Lane 5, 1 ug reshaped VVI Ab, Lane 6, 0.5 ug reshaped VVI Ab.

	10v	20v	30v	40v	50v
VVIHUVH	QVQLQESGPG	LVRPSQTL	SLTCTVSGY	TFSRYWMH	NVRQPPGRGLEWIGYI
	V.LQESG:.L.:P:.....C..SGYTF:RYWMH	NV:Q.PG:GLEWIGYI			
VVIVH	VXLQESGAELAKPGASVKMSCKASGYTF	RYWMH	NVKQRP	GGGLEWIGYI	
	10^	20^	30^	40^	50^
	60v	70v	80v	90v	100v
VVIHUVH	NPSTGYTEYNQKFKD	RVTMLADTSKNQFSLRLSSVTAADTAVYYCARS	SDYR		
	NPSTGYTEYNQKFKD:.T: AD.S.: :LSS:T:.D:AVYYCARS	SDYR			
VVIVH	NPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCARS	SDYR		
	60^	70^	80^	90^	100^
	110v				
VVIHUVH	NWYFDVWGQGT	TTVTVSS			
	NWYFDVWGQGT	TTVTVSS			
VVIVH	NWYFDVWGQGT	TTVTVSS			
	110^				
	60v	70v	80v	90v	100v
VVIHUVK	QQLQVRGSQXQAXGLDIYMGDNDIHFAFLSTGVHSDIQLTQSPSSLSASVG				
				DIQLTQSP: :SAS G	
VVIVK				DIQLTQSPAIMSASPG	
				10^	
	110v	120v	130v	140v	150v
VVIHUVK	DRVTITCSASSSVNYMH	WYQOKPGKAPKLLIYDTSKLAS	GVPSR	FGSGSG	
	::VT:TCSASSSVNYMH	WYQOK:G.:PK IYDTSKLAS	GVP:RFGSGSG		
VVIVK	EKVTMTCSASSSVNYMH	WYQOKSGTSPKRWIYDTSKLAS	GVPAR	FGSGSG	
	20^	30^	40^	50^	60^
	160v	170v	180v	190v	200v
VVIHUVK	TDFTFTISSLPEDIATYYCQWSRNPF	TFGQGTKVEIKREXNLF	PASSVG		
	T:::TISS:::ED ATYYCQWSRNPF	TFG GTK:EI.			
VVIVK	TSYSLTISSMEAEDAATYYCQWSRNPF	TFGSGTKLEIX			
	70^	80^	90^	100^	

FIGURE 24

Comparisons of the amino acid sequences of reshaped VVI (VVIHuVH and VVIHuVK) and murine VVI (VVIVH and VVIVK) antibodies.